EXACT ENGLISH LANGUAGE

TRANSLATION OF THE PCT

APPLICATION AS

ORIGINALLY FILED

WITH ABSTRACT

SPECIFICATION

G-CSF-CONTAINING SUBSTANCE FOR FIBROBLAST RECRUITMENT AND G-CSF-CONTAINING THERAPEUTIC AGENT FOR WOUND HEALING

5 TECHNICAL FIELD

The present invention relates to a substance for recruiting fibroblast, which comprises granulocyte colony-stimulating factor (G-CSF) as an active ingredient. The present invention also relates to a substance for

10 engrafting fibroblast, which comprises G-CSF as an active ingredient. The present invention further relates to a therapeutic agent for healing wound, which comprises G-CSF as an active ingredient.

15 BACKGROUND ART

Human G-CSF is a hematopoietic factor found as a differentiation-inducing factor for hematopoietic stem cells of the granulocytic lineage and is clinically used as a therapeutic agent for neutropenia following bone marrow

20 transplantation or cancer chemotherapy because it promotes *in vivo* hematopoiesis of neutrophils. In addition to this action, human G-CSF acts on stem cells to stimulate their differentiation and proliferation, and also recruits stem cells from the bone marrow into the peripheral blood.

25 Based on the latter action, in fact, transplantation of the peripheral blood hematopoietic stem cells recruited by human G-CSF, i.e., peripheral blood stem cell transplantation is performed in a clinical setting, with

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the aim of facilitating hematopoietic recovery in cancer
patients after intensive chemotherapy.

DISCLOSURE OF THE INVENTION

5   The object of the present invention is to achieve
simple recruitment of fibroblasts into wounded tissues and
engraftment of the fibroblasts in the wounded tissues,
thereby healing the wounds without requiring fibroblast
transplantation.

10   The inventors of the present invention have examined
the regeneration of wounded tissues following myocardial
infarction. As a result, the inventors have found that
upon G-CSF administration, fibroblasts migrate into
myocardial infarct lesions and prevent a reduction in

15   cardiac function, thus alleviating cardiac remodeling.
This finding led to the completion of the present invention.
        Namely, the present invention provides a substance
for recruiting fibroblast, which comprises granulocyte
colony-stimulating factor (G-CSF) as an active ingredient.

20   The present invention also provides a substance for
engrafting fibroblast in a heart after the onset of heart
disease, which comprises G-CSF as an active ingredient.
        The present invention also provides a therapeutic
agent for healing wound, which comprises G-CSF as an active

25   ingredient.
        The present invention further provides a method for
recruiting fibroblast, which comprises administering an
effective amount of G-CSF.

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The present invention also provides a method for engrafting fibroblast in a heart after the onset of heart disease, which comprises administering an effective amount of G-CSF.

5   The present invention also provides a method for healing wound, which comprises administering an effective amount of G-CSF.

When G-CSF was administered after myocardial infarction, a large number of bone marrow cell-derived
10   myocardial cells were observed in infarct lesions. On the other hand, a nearly 10-fold higher number of fibroblasts were also observed. This indicates that G-CSF administration induced migration of various stem cell-derived cells from the bone marrow into infarct lesions,
15   allowed regeneration of the infarct lesions and prevented remodeling following myocardial infarction. G-CSF administration appears to facilitate wound healing and to alleviate remodeling through not only infiltration of many leukocytes during the acute stage, but also migration of
20   fibroblasts into infarct lesions during the chronic stage (60 days after infarction). It has been reported that when fibroblasts were transplanted into infarct lesions following myocardial infarction, remodeling of the infarct lesions could be prevented (Hutcheson KA, Atkins BZ, Hueman
25   MT, Hopkins MB, Glower DD, Taylor DA, Transplant. 9, 2000, 359-368). In the present invention, G-CSF can be administered after myocardial infarction so as to induce migration of fibroblasts from the bone marrow. Thus, the

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present invention can prevent remodeling of infarct lesions without requiring fibroblast transplantation, and hence is very useful for clinical applications.

This fact also means that G-CSF can be clinically

5   applied during various wound healing processes for trauma and so on, in addition to myocardial infarction. Namely, when G-CSF is administered during the wound healing process, not only early infiltration of granulocytes, but also supply of fibroblasts can be caused to accelerate wound

10 healing, thus resulting in a healed tissue that is much stronger. It has previously been considered that G-CSF administration during wound healing causes infiltration of many granulocytes and leads to tissue destruction (Romson, JL, Hook BG, Kunkel SL, Abrams GD, Schork MA, Lucchesi BR,

15 Circulation 67(5), 1983, 1016-1023). However, in the present invention, G-CSF administration allows a stronger healing of wounded sites.

BRIEF DESCRIPTION OF DRAWINGS

20   Figure 1 shows the results of FACS analysis performed on peripheral blood nucleated cells at 60 days after bone marrow transplantation.

Figure 2 shows a confocal laser scanning microscopic photograph of a bone marrow cytospin specimen. Green

25 signals represent GFP-positive bone marrow-derived cells, while blue signals represent nucleated cells visualized by DAPI staining.

Figure 3 shows the survival rate after creation of

myocardial infarction, measured for mice administered

subcutaneously with G-CSF for 10 days (G-CSF group) and

mice administered with physiological saline (control group).

Figure 4 shows transthoracic M-mode echocardiographic

5   images at 60 days after creation of myocardial infarction.

From the top, the panels represent the left ventricles of

normal, control and G-CSF (100 µg/kg)-receiving mice,

respectively.

Figure 5 is a graph showing the effect of G-CSF

10   administration on left ventricular ejection fraction at

60 days after creation of myocardial infarction. From the

left, the bars show the results of the normal group, the

G-CSF group (100 µg/kg) and the G-CSF group (300 µg/kg),

respectively.

15   Figure 6 is a graph showing the effect of G-CSF

administration on left ventricular end-diastolic diameter

at 60 days after creation of myocardial infarction. From

the left, the bars show the results of the normal group,

the G-CSF group (100 µg/kg) and the G-CSF group (300 µg/kg),

20   respectively.

Figure 7(a) shows Azan-stained, short-axis

cross-sections of the heart left ventricles from the

control group (left panel) and the G-CSF group (right

panel) at 60 days after creation of myocardial infarction.

25   Areas stained in red represent muscle fibers, while areas

stained in blue represent collagen fibers.

Figure 7(b) shows a short-axis cross-section of the

heart left ventricle from the G-CSF group at 60 days after

creation of myocardial infarction, as analyzed by Azan
staining (left panel) and under a confocal laser scanning
microscope (right panel).

Figure 8 shows confocal laser scanning microscopic
photographs of myocardial infarct lesions: (a) non-
infarcted site; (b) myocardial infarct lesion in the
control group; (c) myocardial infarct lesion in the G-CSF
group (100 µg/kg); and (d) myocardial infarct lesion in
Lac-Z transgenic mice administered with G-CSF (100 µg/kg).

Figure 9 is a graph showing the number of
GFP-positive cells present in myocardial infarct lesions.

Figure 10 shows confocal laser scanning microscopic
photographs of a single myocardial infarct lesion
immunostained with anti-α-smooth muscle actin antibody.
Green signals represent GFP-positive cells, blue signals
represent DAPI-stained nuclei, and red signals represent
α-smooth muscle actin.

Figure 11 shows confocal laser scanning microscopic
photographs of a single myocardial infarct lesion
immunostained with anti-von Willebrand factor (vWF)
antibody. Green signals represent GFP-positive cells, blue
signals represent DAPI-stained nuclei, and red signals
represent vWF.

Figure 12 shows microscopic photographs of a single
myocardial infarct lesion immunostained with anti-actinin
antibody. Green signals represent GFP-positive cells, blue
signals represent DAPI-stained nuclei, and red signals
represent actinin.

Figure 13 shows microscopic photographs of a single myocardial infarct lesion immunostained with anti-actinin antibody. Green signals represent GFP-positive cells, blue signals represent DAPI-stained nuclei, and red signals

5   represent actinin.

Figure 14 shows microscopic photographs of a single myocardial infarct lesion sliced in 1 μm sections and immunostained with anti-actinin antibody. Green signals represent GFP-positive cells, blue signals represent

10   DAPI-stained nuclei, and red signals represent actinin.

Figure 15 is a graph showing percentages of GFP-positive cells by type in myocardial infarct lesions after G-CSF administration.

Figure 16 is a graph showing the effect of G-CSF

15   administration on the number of peripheral blood nucleated cells in both the group receiving whole bone marrow transplantation and the group receiving single hematopoietic stem cell transplantation.

Figure 17 is a view showing the effect of G-CSF

20   administration on survival rate after creation of myocardial infarction. In the figure, the dotted line represents the group receiving whole bone marrow transplantation, the solid line represents the group receiving single hematopoietic stem cell transplantation,

25   solid and open squares represent the group treated with G-CSF (+), and open and solid circles represent the group untreated with G-CSF (-).

Figure 18 shows confocal laser scanning microscopic

photographs of myocardial infarct lesions immunostained with anti-vimentin antibody in the group receiving single hematopoietic stem cell transplantation. Green signals represent GFP-positive cells, blue signals represent

5   DAPI-stained nuclei, and red signals represent vimentin.

Figure 19 is a graph showing the effect of G-CSF administration on the number of GFP-positive cells in both the group receiving whole bone marrow transplantation and the group receiving single hematopoietic stem cell

10   transplantation.

Figure 20 is a confocal laser scanning microscopic photograph showing the boundary area of myocardial infarction, immunostained with anti-actinin antibody, in the group receiving single hematopoietic stem cell

15   transplantation when administered with G-CSF. Green signals represent GFP-positive cells, blue signals represent DAPI-stained nuclei, and red signals represent actinin.

20   BEST MODE FOR CARRYING OUT THE INVENTION

The present invention relates to a substance for recruiting fibroblast, which comprises G-CSF as an active ingredient. There is no particular limitation on the site where fibroblasts are recruited. For example, in a case

25   where there is a wounded tissue, fibroblasts can be recruited into the wounded tissue by G-CSF administration.

As used herein, the term "wound" or "wounded" refers to damage or injury to body tissues. Examples include

damage or injury to internal tissues or organs (e.g., heart, lung, kidney, intestine, liver, tendon), as well as trauma to the skin, etc. As specific examples of wounded tissues, myocardial infarcted hearts can be preferably presented.

5 In general, fibroblasts are unique to connective tissue and have oval nuclei and spindle-shaped protoplasm, characterized by good development of rough endoplasmic reticulum and Golgi apparatus. Moreover, fibroblasts also include mesenchymal lineage cells which are found in many

10 organs and play a role in parenchymal filling. Fibroblasts generally have the ability to produce large amounts of interstitial substances (e.g., collagen, fibronectin, mucopolysaccharides) in the body. Fibroblast recruitment into wounded tissues enables the facilitation of wound

15 healing.

The present invention also relates to a substance for engrafting fibroblast in a heart after the onset of heart disease, which comprises G-CSF as an active ingredient.

Specific examples of heart diseases include ischemic

20 heart diseases (e.g., myocardial infarction) and myocardial diseases (e.g., cardiomyopathy). For example, G-CSF administration after myocardial infarction enables fibroblasts engraftment into myocardial infarct lesions.

The present invention further relates to a

25 therapeutic agent for healing wound, which comprises G-CSF as an active ingredient.

G-CSF used in the present invention is not limited in any way, but a highly purified one is preferred. Specific

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examples include mammalian G-CSF, particularly those having

substantially the same biological activities as human G-CSF.

G-CSF used herein may be of any origin, including those

naturally occurring and those produced recombinantly, with

5   recombinantly produced G-CSF being preferred. Such

recombinantly produced G-CSF may have the same amino acid

sequence as naturally-occurring G-CSF or may comprise

deletion, substitution and/or addition of one or more amino

acid residues in the amino acid sequence as long as it

10   retains the same biological activities as naturally-

occurring G-CSF. Amino acid deletions, substitutions or

additions may be accomplished in a manner known to those

skilled in the art. For example, those skilled in the art

will be able to use site-specific mutagenesis (Gotoh, T. et

15   al. (1995) Gene 152, 271-275; Zoller, M.J. and Smith, M.

(1983) Methods Enzymol. 100, 468-500; Kramer, W. et al.

(1984) Nucleic Acids Res. 12, 9441-9456; Kramer, W. and

Fritz, H.J. (1987) Methods Enzymol. 154, 350-367; Kunkel,

T.A. (1985) Proc. Natl. Acad. Sci. USA. 82, 488-492; Kunkel

20   (1988) Methods Enzymol. 85, 2763-2766) or other techniques

to introduce appropriate mutations into the amino acid

sequence of G-CSF, thereby preparing a polypeptide

functionally equivalent to G-CSF. Likewise, amino acid

mutations may also occur in the natural world. In general,

25   amino acid residues to be substituted are preferably

replaced with other amino acids in such a manner as to

conserve the nature of amino acid side chains. With regard

to the nature of amino acid side chains, examples include

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hydrophobic amino acids (A, I, L, M, F, P, W, Y, V),
hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, T),
amino acids having an aliphatic side chain (G, A, V, L, I,
P), amino acids having a hydroxy-containing side chain (S,
5   T, Y), amino acids having a sulfur-containing side chain (C,
M), amino acids having a carboxylic acid- or amide-
containing side chain (D, N, E, Q), amino acids having a
base-containing side chain (R, K, H), and amino acids
having an aromatic-containing side chain (H, F, Y, W)
10   (capital letters in parentheses refer to the corresponding
amino acids in single-letter notation). It is already
known that a polypeptide having an amino acid sequence
modified from another amino acid sequence by deletion
and/or addition of one or more amino acid residues and/or
15   by their substitution with other amino acids retains the
same biological activities as the original polypeptide
(Mark, D.F. et al., Proc. Natl. Acad. Sci. USA (1984) 81,
5662-5666; Zoller, M.J. & Smith, M. Nucleic Acids Research
(1982) 10, 6487-6500; Wang, A. et al., Science 224, 1431-
20   1433; Dalbadie-McFarland, G. et al., Proc. Natl. Acad. Sci.
USA (1982) 79, 6409-6413).

Moreover, in the present invention, G-CSF may be
administered as a protein, but it is also possible to
administer a gene encoding G-CSF, as in gene therapy. The
25   gene encoding G-CSF is generally administered, for example,
as an expression vector containing an expression cassette.
The vector is not limited in any way, and either a non-
virus or virus vector may be used (e.g., Supplementary

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Volume of Experimental Medicine, "Experimental Methods for Gene Transfer and Expression Analysis," YODOSHA, 1996). Examples of the vector include a plasmid vector, a virus vector, a phage vector, a cosmid vector and a YAC vector.

5 The expression vector normally includes a regulatory element, such as a promoter. Any method is available for gene transfer, and include, for example, calcium phosphate transfection, lipofection, a method using a liposome, the naked-DNA method, receptor-mediated gene transfer, a method

10 using a gene gun, DEAE-dextran transfection, and a method using a capillary tube. In the present invention, the gene may be directly transferred into the body, or after gene transfer into cells taken up from the body, the cells may be returned into the body.

15 It is also possible to use fusion proteins between G-CSF and other proteins. To prepare a fusion polypeptide, for example, DNA encoding G-CSF and DNA encoding another protein may be ligated together in-frame, introduced into an expression vector and then expressed in a host. Other

20 proteins to be fused herein with G-CSF are not limited in any way.

Moreover, it is also possible to use chemically modified G-CSF. Examples of chemically modified G-CSF include those modified by structural alteration, addition

25 and/or deletion of sugar chains, as well as those conjugated with a compound such as an inorganic or organic compound (e.g., polyethylene glycol, vitamin B12).

G-CSF used in the present invention may be prepared

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in any manner, for example, by culturing human tumor cell lines or human G-CSF-producing hybridoma cell lines or by genetically engineered production in *E. coli* cells, yeast cells, Chinese hamster ovary (CHO) cells, C127 cells, COS cells, myeloma cells, BHK cells, insect cells, etc. G-CSF thus prepared is extracted, isolated and purified in various manners before use. G-CSF used in the present invention is preferably a genetically engineered one, more preferably G-CSF produced in mammalian cells (particularly CHO cells) (e.g., JP 1-44200 B, JP 2-5395 B, JP 62-129298 A, JP 62-132899 A, JP 62-236488 A, JP 64-85098 A).

If necessary, depending on the administration mode and the dosage form, the substances of the present invention for fibroblast recruitment or other purposes may be supplemented as appropriate with a suspending agent, a solubilizing agent, a stabilizing agent, an isotonizing agent, a preservative, an anti-adsorption agent, a surfactant, a diluent, an excipient, a pH adjustor, a soothing agent, a buffer, a sulfur-containing reducing agent, an antioxidant or the like.

Examples of a suspending agent include methylcellulose, Polysorbate 80, hydroxyethylcellulose, gum arabic, powdered tragacanth, carboxymethylcellulose sodium, and polyoxyethylenesorbitan monolaurate.

Examples of a solubilizing agent include polyoxyethylene hydrogenated castor oil, Polysorbate 80, nicotinamide, polyoxyethylenesorbitan monolaurate, Magrogol, and an ethyl ester of castor oil fatty acid.

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Examples of a stabilizing agent include Dextran 40, methylcellulose, gelatin, sodium sulfite, and sodium metasulfite.

Examples of an isotonizing agent include D-mannitol and sorbitol.

Examples of a preservative include methyl paraoxybenzoate, ethyl paraoxybenzoate, sorbic acid, phenol, cresol, and chlorocresol.

Examples of an anti-adsorption agent include human serum albumin, lecithin, dextran, ethylene oxide-propylene oxide copolymers, hydroxypropylcellulose, methylcellulose, polyoxyethylene hydrogenated castor oil, and polyethylene glycol.

Examples of a sulfur-containing reducing agent include those having a sulfhydryl group such as N-acetylcysteine, N-acetylhomocysteine, thioctic acid, thiodiglycol, thioethanolamine, thioglycerol, thiosorbitol, thioglycolic acid and a salt thereof, sodium thiosulfate, glutathione, and a C_1-C_7 thioalkanoic acid.

Examples of an antioxidant include erythorbic acid, dibutylhydroxytoluene, butylhydroxyanisole, α-tocopherol, tocopherol acetate, L-ascorbic acid and a salt thereof, L-ascorbyl palmitate, L-ascorbyl stearate, sodium bisulfite, sodium sulfite, triamyl gallate and propyl gallate, as well as chelating agents such as ethylenediaminetetraacetic acid disodium salt (EDTA), sodium pyrophosphate and sodium metaphosphate.

Furthermore, the substances of the present invention

for fibroblast recruitment or other purposes may comprise
other commonly used ingredients, e.g., inorganic salts such
as sodium chloride, potassium chloride, calcium chloride,
sodium phosphate, potassium phosphate and sodium
5 bicarbonate, as well as organic salts such as sodium
citrate, potassium citrate and sodium acetate.

The substances of the present invention for
fibroblast recruitment or other purposes may be
administered in a dosage form of injections (e.g.,
10 subcutaneous, intracutaneous, intramuscular, intravenous or
intraperitoneal injections), in any dosage form suitable
for transdermal, transmucosal or transnasal administration
or in any dosage form suitable for oral administration
(e.g., tablets, capsules, granules, solutions, suspensions).
15 The present invention is not limited by the route of
administration or dosage form, etc.

In the substances of the present invention for
fibroblast recruitment or other purposes which comprises
G-CSF as an active ingredient, the dose and frequency of
20 its administration can be determined as appropriate by
those skilled in the art by taking into account symptoms of
a diseased patient to be treated. In general, G-CSF may be
administered at a dose of 0.1 to 500 µg/kg/day per adult,
preferably 1 to 50 µg/kg/day per adult, for 1 to 7 days per
25 week. However, the present invention is not limited by the
dose of G-CSF. Alternatively, the substances of the
present invention for fibroblast recruitment or other
purposes may be used in combination with other

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pharmaceutical agents.

EXAMPLES

Example 1

5   Whole bone marrow transplantation

(1) Creation of murine bone marrow transplantation model

C57BL/6 mice at 8 to 10 weeks of age (CLEA, Tokyo,

Japan) were irradiated once with a lethal dose of total

body irradiation (850 cGy) using a 4×10^6 V linear

10 accelerator and used as recipient mice. Bone marrow crude

fractions were collected from the femurs and neck bones of

GFP transgenic mice (C57BL/6, 10-12 weeks of age) (Okabe et

al., (1997) FEBS. Lett. 407, 313-319), and 5×10^6 bone

marrow cells were transplanted into the recipient mice

15 through their tail veins.

To confirm what percentage of donor-derived GFP-

positive bone marrow cells have been engrafted in the bone

marrow of the recipients (this percentage being hereinafter

referred to as "chimera rate"), peripheral blood nucleated

20 cells obtained at 60 days after bone marrow transplantation

were analyzed using a FACS Calibur (Becton Dickinson, San

Jose, CA, USA). Figure 1 shows the results obtained. In

the control mice, GFP expression was not observed in their

bone marrow nucleated cells, whereas cells in the bone

25 marrow-transplanted mice were GFP-positive at a rate of

97.8%. The degree of chimera rate is an important index in

quantifying donor-derived GFP-positive cells. In this

experiment, mice whose chimera rate of peripheral blood

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cells averaged 95% or more were used as a murine bone
marrow transplantation model. This is considered as being
able to satisfy the requirement for sufficient
quantification and evaluation of the contribution of bone
5   marrow-derived cells to wound healing.

Figure 2 shows a cytospin specimen of the bone marrow,
which was stained with DAPI and observed under a confocal
laser scanning microscope. As a result, most of the
nucleated cells (blue) were visualized in green, indicating
10   that the bone marrow cells were GFP-positive.
(2) Effect of G-CSF administration on myocardial infarction
mice

At 60 days after bone marrow transplantation, the
mice were intubated and anesthetized with 0.5% isoflurane
15   gas. After thoracotomy, the left ventricle was exposed and
the left coronary artery was ligated to create myocardial
infarction. At 24 hours after creation of myocardial
infarction, the mice were subcutaneously administered with
recombinant human G-CSF (100 or 300 μg/kg/day; Chugai
20   Pharmaceutical Co., Ltd., Japan) dissolved in physiological
saline once a day for 10 consecutive days (G-CSF group).
Mice in the control group received physiological saline
alone.
Survival rate

25   The survival rate was examined for both the G-CSF
group (300 μg/kg) and the control group (n = 68) (Figure 3).
The survival rate of the control group was about 60% at
60 days after creation of myocardial infarction, whereas

the survival rate of the G-CSF group was about 90%.

Morphological observation

At 60 days after creation of myocardial infarction, transthoracic echocardiography (M-mode echocardiography) was performed on normal mice and mice in the control group and in the G-CSF group by using image point 1500 ultrasonic diagnostic equipment (Philips Co., USA) provided with a 15 MHz phased array transducer, followed by morphological observation of myocardial infarct lesions. The mice were anesthetized with ketamine (30 mg/kg) and xylazine (6 mg/kg) to maintain spontaneous respiration. As shown in Figure 4, when compared to the normal left ventricle, the control group had thinned and non-constrictive myocardium in the anterior wall, along with an increased left ventricular inner diameter. In contrast, in the G-CSF group (100 µg/kg), such an increase in the left ventricular end-diastolic diameter was prevented when compared to the control group. Moreover, the left ventricular anterior wall in the G-CSF group was low constrictive, but significantly improved when compared to the control group.

Cardiac function

At 60 days after creation of myocardial infarction, the left ventricular end-systolic diameter (LVESD) and end-diastolic diameter (LVEDD) were determined from the M-mode images of the control group and the G-CSF group (100 or 300 µg/kg) (n = 68). Their end-diastolic volume (EDV) and end-systolic volume (ESV) were also calculated by the Teichholz method. The left ventricular ejection fraction

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(EF) was calculated according to the following equation.

EF (%) = [(EDV-ESV)/EDV] × 100

The results obtained are shown in Figures 5 and 6. Both figures indicated that the G-CSF groups showed a significant improvement in their cardiac function.

Histological observation

(i) Preparation of sections

The mice were anesthetized with ketamine (30 mg/kg) and xylazine (6 mg/kg). Their hearts were perfused with PBS and perfusion-fixed with 4% paraformaldehyde in PBS. The hearts were excised, embedded in OCT compound (Miles Scientific, Naperville, IL, USA) and quickly frozen in liquid nitrogen. The embedded hearts were sliced to prepare sections.

(ii) Azan staining

At 60 days after creation of myocardial infarction, Azan staining was performed on the frozen short-axis cross-sections of the heart left ventricles from the control group and the G-CSF group (300 μg/kg). The results obtained are shown in Figure 7(a). The control group showed an increase in its left ventricular diameter, along with infarct "thinning" and infarct expansion, which means so-called remodeling following myocardial infarction. In contrast, the G-CSF group showed mild post-infarction remodeling, and infarct "thinning" and infarct expansion were reduced. This indicated that G-CSF administration allowed tissue regeneration in myocardial infarct lesions and hence prevented remodeling.

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Furthermore, the sections from the G-CSF group were observed under a confocal laser scanning microscope, indicating that a large number of GFP-positive bone marrow cells were infiltrated into myocardial infarct lesions

5   (Figure 7(b), right panel).

(iii) Immunostaining

The frozen sections (6 μm) were rinsed with PBS and stained overnight at 4°C using a specific antibody. The sections were then rinsed three times with PBS and

10   incubated with a TRITC (DAKO, Japan)-conjugated secondary antibody at 4°C for 4 hours (red). The nuclei were stained with DAPI (Sigma Aldrich) (blue).

Myocardial infarct lesions were observed under a confocal laser scanning microscope (LSM410; Carl Zeiss,

15   Jena, Germany) (Figure 8). The sections of non-infarcted sites were stained with anti-α-actinin antibody (clone EA-53; Sigma Aldrich, Saint Louis, MO, USA). Myocardial cells were stained in red and a few GFP-positive cells remained within the blood vessels (a). The control group

20   only showed a few GFP-positive cells in its infarct lesions (b). In contrast, the G-CSF group (300 μg/kg) showed a large number of GFP-positive cells in its infarct lesions (c). Using Lac-Z transgenic mice (10-12 weeks of age; Jackson Laboratories, Bar Harbor, ME, USA) as donor mice,

25   the same experiment as used for (c) was repeated to confirm that the green fluorescence observed in the infarct lesions of (c) was not non-specific fluorescence (d). In addition, images obtained with the confocal laser scanning microscope

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were transferred to a computer and analyzed with NIH image
software. The number of GFP-positive cells was calculated
relative to the number per unit area of cells present in
infarct lesions of each group. The results obtained are
5   shown in Figure 9. It was indicated that upon G-CSF
administration, bone marrow-derived cells migrated to
myocardial infarct lesions.

Next, a further experiment was performed to confirm
what type of cells these GFP-positive cells were.

10   Figure 10 shows the results of immunostaining with
anti-α-smooth muscle actin antibody (clone 1A4; Sigma
Aldrich). The bone marrow-derived GFP-positive cells were
stained in red, indicating that they were differentiated
into smooth muscle cells.

15   Next, to study the cells for von Willebrand factor
(vWF) which was an endothelial cell marker, immunostaining
was performed with anti-vWF antibody (clone F8/86; DAKO)
(Figure 11). The results showed areas in which GFP signals
were surrounded by vWF signals, indicating that the
20   GFP-positive cells were also differentiated into
endothelial cells.

Further, myocardial cells were immunostained with
anti-α-actinin antibody·(Figures 12 to 14). As shown in
Figures 12 and 13, actinin-positive signals were observed
25   among GFP-positive cells, some of which showed a cross
striation pattern and were suggested to be myocardial cells.
Myocardium having a perfect cross striation pattern was
regenerated from GFP-positive cells. Figure 14 shows

photographs of sections sliced in 1 μm increments. The
results indicated that bone marrow-derived GFP-positive
cells achieved complete regeneration of myocardial cells.

Figure 15 summarizes the percentages of myocardial
5   cells, vascular endothelial cells, smooth muscle cells and
fibroblasts among GFP-positive cells observed in myocardial
infarct lesions at 60 days after creation of myocardial
infarction. The number of cells was determined as follows.
Each heart was divided into three parts, i.e., apex, middle
10 and base, and the volume of infarcted tissue was calculated
from the area of GFP-positive cells and the tissue
thickness in each part. The number of GFP-positive cells
per unit area was counted to determine the density of
GFP-positive cells. The density of GFP-positive cells and
15 the infarct volume were used to calculate the number of
GFP-positive cells in the infarcted tissue. At least 80%
of the cells were spindle-shaped and had different
morphology than hematopoietic cells. Moreover, these cells
were also found to be CD45-negative and Mac-1-negative.
20 This indicated that fibroblasts were the largest in number
among GFP-positive cells present in myocardial infarct
lesions.

Example 2
25 Transplantation of bone marrow-derived single hematopoietic
stem cells

Bone marrow was collected from GFP transgenic mice
and a GFP-positive fraction was separated with a cell

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sortor, followed by collection of c-kit-positive, Sca-1-positive, linage antigen-negative and CD34-negative hematopoietic stem cells. A single cell selected from these cells and 5 × 10^6 cells of a bone marrow crude

5   fraction collected from another normal donor mouse were transplanted into the bone marrow of lethally irradiated recipient mice. After 3 months, the rate of GFP-positive cells engrafted in the bone marrow was confirmed. After thoracotomy under anesthesia, the left coronary artery was

10   ligated to create myocardial infarction. The mice were then subcutaneously administered with G-CSF (300 µg/kg) for 10 days. The number of peripheral blood nucleated cells at 10 days after G-CSF administration was about 30,000 in both the group receiving whole bone marrow transplantation and

15   the group receiving single hematopoietic stem cell transplantation; there was no difference between these two groups (Figure 16).

The survival rate was examined for both the group receiving single hematopoietic stem cell transplantation

20   (solid line) and the group receiving whole bone marrow transplantation (dotted line) when administered with (square) or without (circle) G-CSF (Figure 17). Not only the group receiving whole bone marrow transplantation, but also the group receiving single hematopoietic stem cell

25   transplantation showed a significant improvement in their survival rate upon G-CSF administration.

Next, the same procedure as used in Example 1 was repeated to prepare sections of myocardial infarct lesions

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from the group receiving single hematopoietic stem cell transplantation, followed by immunostaining with anti-vimentin antibody (PROGEN BIOTECHNIK GMBH, Cat. No. GP53). As shown in Figure 18, the presence of GFP-positive cells

5   was observed in myocardial infarct lesions. The results indicated that a single GFP-positive hematopoietic stem cell was engrafted in a myocardial infarct lesion. Moreover, this cell was found to be stained with anti-vimentin antibody, confirming that it was differentiated

10   into a fibroblast.

Further, the same procedure as used in Example 1 was repeated to determine the number of GFP-positive cells in both the group receiving single hematopoietic stem cell transplantation and the group receiving whole bone marrow

15   transplantation when administered with or without G-CSF (Figure 19). Not only the group receiving whole bone marrow transplantation, but also the group receiving single hematopoietic stem cell transplantation showed a significant increase in the number of GFP-positive cells

20   upon G-CSF administration.

Immunostaining with anti-myocardial actinin antibody was performed on left ventricular sections from the group receiving single hematopoietic stem cell transplantation when administered with G-CSF (Figure 20). GFP-positive

25   cells were found to be actinin-positive, indicating that they were differentiated into myocardial cells.

In Examples 1 and 2 above, all values were expressed as mean ± SEM. Statistical significance between means was

calculated by ANOVA. Comparison between the control group and the G-CSF group was made by log-rank assay or non-parametric Fisher's multiple comparison test. p<0.05 was considered as statistically significant.

5

Example 3

Lethally irradiated C57BL/6 mice (8-10 weeks of age) were transplanted through their tail veins with whole bone marrow cells (w-BM) collected from the bone marrow of CAG-
10   EGFP mice (Okabe M. et al., FEBS Lett. 1997, 407: 313-319) or with c-kit-positive, Sca-1-positive and lineage antigen-negative single population cells (KSL-SP). After 8 weeks, the left coronary artery was ligated to create myocardial infarction (MI) in each mouse. At 24 hours after MI
15   creation, the mice were subcutaneously administered with physiological saline (G-CSF(-)) or 300 µg/kg/day G-CSF (G-CSF(+)) once a day for 10 consecutive days. At 8 weeks after MI creation, the mice were dissected and their hearts were analyzed immunohistologically. For each mouse group
20   (n = 10), 100 sample specimens were counted for the numbers of GFP-positive cells, GFP-positive and vimentin-positive cells, as well as GFP-positive and actinin-positive cells in infarct lesions. Table 1 shows average numbers of these GFP-positive cells.

25

Table 1 Quantitative analysis of GFP-positive cells in
          infarct lesions

Mouse	GFP-positive cells		
	Total*	Vimentin positive	Actinin positive
w-BM G-CSF (-)	8841	1813	65
w-BM G-CSF (+)	119802	37457	5403
KSL-SP G-CSF (-)	1224	480	0
KSL-SP G-CSF (+)	41779	9322	3

*: Total number of GFP-positive cells in infarct lesions

In the w-BM groups, both vimentin-positive cells
(fibroblasts) and actin-positive cells (myocardial cells)
were regenerated from the whole bone marrow, whereas in the
KSL-SP groups, only fibroblasts were regenerated. This
result suggests that myocardial cells are not regenerated
from hematopoietic stem cells. Myocardial cell
regeneration is considered to be mediated by
differentiation from stem cells of the mesenchymal lineage.

Next, when attention is directed to the effect of
G-CSF, G-CSF was found to recruit fibroblasts and
myocardial cells in the w-BM group. In the KSL-SP group,
G-CSF was found to recruit fibroblasts, but only three
myocardial cells were observed in this group. These
myocardial cells probably appear to be a result of cell
fusion, but not regeneration.

In all the groups, as shown in Figure 17, G-CSF
administration provides an improvement in survival rate.
These effects are mediated by recruitment of fibroblasts

and myocardial cells in the w-BM group, whereas they are mediated by recruitment of fibroblasts in the KSL-SP group. In particular, an increase in the number of fibroblasts contributes to wound healing at myocardial infarct sites and facilitates the prevention of remodeling known to be correlated with mortality.

INDUSTRIAL APPLICABILITY

Simply by using the substance for fibroblast recruitment according to the present invention to induce migration of a few bone marrow-derived cells, it is possible to regenerate tissues such as myocardial infarct lesions and improve the survival rate without performing fibroblast transplantation. Moreover, the use of the therapeutic agent for wound healing according to the present invention enables a strong healing of wounded sites.